Thrombospondin-1 induces matrix metalloproteinase-2 activation in vascular smooth muscle cells

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Introduction: **Thrombospondin-1 (TSP-1), an extracellular matrix (ECM) glycoprotein, is associated with a variety of cellular processes relevant to atherosclerosis and intimal hyperplasia, including vascular smooth muscle cell (VSMC) migration. Matrix metalloproteinase-2 (MMP2) is associated with basement membrane and ECM degradation, important processes for cell migration. We hypothesized that TSP-1 modulates MMP2 activity in VSMCs and is critical for VSMC migration.**
Methods: **Quiescent bovine aortic VSMCs (48 hours) were incubated in serum-free media (SFM) with or without TSP-1 (10 or 20 μg/mL). Gelatinase activity was measured with zymography to determine pro-MMP2 and MMP2 activity. MMP2 messenger RNA expression was determined with Northern blot analysis. Invasion assays were performed. A binding assay was used to determine the specificity of TSP-1 binding to MMP2. Blots were quantified with densitometry, and all comparisons were made with a paired *t* test.**
Results: **TSP-1 induced production of activated forms of MMP2, as well as upregulation of pro-MMP2. MMP2 mRNA was upregulated 1.7-fold by TSP-1 at 10 and 20 μg/mL. GM6001, an MMP inhibitor, inhibited VSMC migration across the matrix barrier, whereas migration that occurred in the absence of the matrix barrier was unaffected. With a binding assay, TSP-1 interacted physically with MMP2, and TSP-1–bound MMP2 showed the strongest binding activity in comparison with collagen I, fibronectin, and elastin.**
Conclusion: **TSP-1 induced MMP2 activation through transcriptional and posttranslational mechanisms. These findings imply that MMP2 activation is relevant to the mechanism of TSP-1–induced VSMC migration. (J Vasc Surg 2003;38: 147-54.)**

Vascular smooth muscle cell (VSMC) migration contributes to development of vascular pathologic states, including intimal hyperplasia and atherosclerotic plaque formation. Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that are able to digest a wide range of extracellular matrix (ECM) proteins, which are contributory to certain processes, eg, during development,[1] vasculitis,[2] and after vascular injury.[3,4] More than 20 members of the MMP family exist.[5] More well-known MMP family members include collagenases (MMP1), which degrade structural types I, II, and III collagen; stromelysins (MMP3), which have a broad substrate specificity; and type IV collagenases, also called gelatinases (MMP2, MMP9). Increasing evidence exists that expression and activation of MMP2 are associated with VSMC migration and proliferation in vitro.[6,7]

Thrombospondin-1 (TSP-1), a matricellular glycoprotein chemotactic for VSMCs, is not integral to arterial wall structure but is expressed locally after acute arterial injury, in atherosclerotic plaque, and in early intimal hyperplasia.[8,9] TSP-1 contains a number of binding sites that have been implicated in interactions with more than 30 cell-surface and matrix proteins, including structural proteins, cell surface receptors, enzymes, and cytokines.[10] TSP-1 has been implicated in a number of biologic processes, including cell migration, coagulation, cell adhesion, cell growth, modulation of cell-cell and cell-matrix interactions, tumor growth and metastases, and angiogenesis, because of its ability to interact with such a wide variety of proteins.[11,12]

Cell-matrix interactions likely have a substantial role in regulation of cellular proteolysis. Various reports have emphasized involvement of ECM components, including fibronectin, bone matrix proteins such as osteopontin, and type I collagen, in induction of pro-MMP2 activation.[13,14] As MMP2 and TSP-1 are involved in VSMC migration, a process associated with development of vascular lesions, we sought to determine whether TSP-1 modulates MMP2 activation in vitro and whether TSP-1–induced migration depends on MMP2.

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MATERIALS AND METHODS

VSMC culture. Bovine aortic VSMCs were isolated and seeded in 10% fetal bovine serum (FBS; Hyclone, Logan, Utah) and 1% antibiotic (Gibco BRL, Gaithersburg, Md) in primary culture.[11] Subconfluent monolayers of VSMCs in early passage (2-5) were used for each experiment. In all experiments, cells were quiescent in serum-free medium (SFM; Dulbecco modified Eagle medium [DMEM]; Gibco BRL) for 48 hours.

Materials. Purified TSP-1, derived from human platelets, was generously provided by Jack Lawler, PhD, Beth Israel Deaconess Medical Center, Boston, Mass. The concentration of TSP-1 used for all invasion and chemotaxis experiments was 20 μg/mL), the peak concentration for cell migration.[15] Matrigel (BD Bioscience, Bedford, Mass), GM 6001 (Chemicon, Temecula, Calif), purified pro-MMP2 (Calbiochem, La Jolla, Calif), and anti-MMP2 antibodies (MMP2[2C1] mouse, monoclonal IgG_1 specific for MMP2; Santa Cruz, Santa Cruz, Calif) were purchased.

Chemotaxis and invasion assay. VSMC invasion was investigated with a modified Boyden chamber (Neuro-Probe, Gaithersburg, MD). Polyvinylidene floride (PVDF) filters were coated with a layer of the reconstituted basement membrane (Matrigel; 500 μg/mL). Lower wells were filled with SFM or 10% FBS containing 0.1% bovine serum albumin (BSA), and coated filters were mounted in the chamber. VSMCs (5×10^5), suspended in SFM or TSP-1 (20 μg/mL), were placed in the upper well and allowed to settle on the coated filters. After 4 or 18 hours (37°C) the membrane was fixed in 70% ethanol and stained with hematoxylin. VSMCs that migrated to the bottom side of the membrane were counted for 5 high-power fields (HPF;, 400×) per well. Each experiment was performed in triplicate. The membrane was left uncoated for chemotaxis experiments.

Northern blot analysis. Total cellular RNA was extracted from TSP-1–stimulated VSMCs with TRIzol reagent (Life Technology, Carlsbad, Calif) in accord with the manufacturer's instructions. RNA concentration was determined with a spectrophotometer. Ten micrograms or 15 μg of total RNA was denatured in sample buffer (20 mmol/L 3-morpholinopropane sulfonic acid, 6% formaldehyde, 50% formamide), electrophoresed through a 1% agarose-formaldehyde gel, and then transferred to a nylon membrane (Boehringer Mannheim, Indianapolis, Ind) by capillary transfer with 10× SSC. Hybridization was performed with probes constructed from the human MMP2 cDNA, labeled with a random priming Oligolabelling Kit (Amersham Bioscience, Piscataway, NJ) with [^{32}P]-deoxycytidine monophosphate (dCTP) and purified on Sephadex-G50 columns (Boehringer Mannheim). Blots were washed twice with 1× SSC, 0.1% SDS (25°C), 0.1× SSC, and 0.1% SDS (65°C), and then were exposed for several hours or as necessary. Blots were stripped and reprobed with a glyceraldehyde 3-phosphate dehydrogenase probe (American Type Culture Collection, Manassas, Va) to standardize for RNA loading.

TSP-1 binding. Microtiter plates (BD Bioscience) were coated overnight by passive adsorption at 4°C in assay buffer (100 mmol/L of Tris-HCl [pH 7.5], 100 mmol/L of NaCl, 10 mmol/L of $CaCl_2$, 0.05% Brij-35) containing 100 μg of the indicated matrix protein, as described.[16] Nonspecific binding sites on coated wells were blocked by incubation with a 200 mL volume of 3% BSA in assay buffer for 2 hours (37°C). To evaluate activity of TSP-1–bound MMP2, purified MMP2 (1 mmol/L) was activated with 2 mmol/L of amino-phenylmercuric acetate (APMA) for 30 minutes (37°C), diluted to 50 nmol/L in assay buffer, and incubated overnight (4°C) in the selected matrix protein (BSA, TSP-1, type I collagen, fibronectin, or elastin)–coated wells in a 50 μL volume. Aliquots (5 mL) were removed and analyzed with gelatin substrate zymography (designated "soluble phase"). Wells were then washed five times with 50 μL of assay buffer, and the bound enzyme was eluted with two different techniques, one using nonreducing Laemmli sample buffer (50 μL for 1 hour at 25°C, designated "bound") and analyzed with gelatin zymography, and the other using reducing Laemmli sample buffer (50 μg, designated "bound") and analyzed with Western blot. For the zymography analysis, the presence of bound MMP2 was confirmed by incubating wells overnight at 4°C with 10% dimethyl sulfoxide (DMSO) to disrupt MMP2-ECM protein interactions.[17]

Western blot analysis. Proteins were separated with sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) and transferred to a nylon membrane (Amersham, Arlington Heights, Ill). Blocking was performed in a 5% non-fat milk–tris-buffered saline solution–Tween-20 (TBST) solution. Blots were incubated in a 5% non-fat milk-TBST solution with anti-MMP2 (Santa Cruz).

Gelatin zymography. Conditioned media were subjected to electrophoresis in 10% SDS-PAGE containing 1 mg/mL of gelatin under nonreducing conditions, as described.[14] After electrophoresis, the gels were washed with 2.5% Triton X-100 to remove SDS and incubated for 48 hours at 37°C in 50 mmol/L of Tris buffer containing 200 nmol/L of NaCl and 20 mmol/L of $CaCl_2$, pH 7.4. The gels were stained with 0.5% Coomasie brilliant blue R-250 in 10% acetic acid and 45% methanol, and were destained.

Statistical analysis. All studies were performed in triplicate. Blots were quantified with densitometry with Un-Scan-It Software (Silk Scientific, Orem, Utah). The data were analyzed with a paired *t* test (SigmaStat; SPSS Science, Chicago, Ill) for chemotaxis, invasion, and densitometry studies. $P < 0.05$ was considered significant.

Experimental design

Three general types of experiments were performed: static culture experiments, invasion assays, and binding assays.

Static culture experiments. These experiments were performed to look for MMP2 activation by TSP-1 without

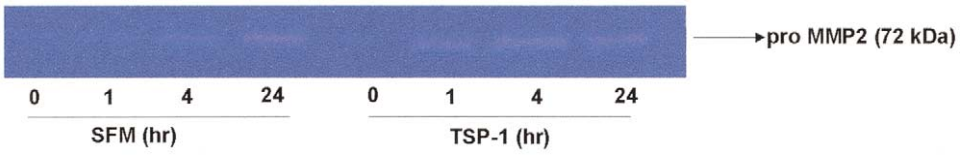

Fig 1. TSP-1 upregulates pro-MMP2. VSMCs were seeded in plates and cultured in 10% FBS for 48 hours, then incubated for 24 hours in SFM. Subsequently, the medium was changed with SFM with or without TSP-1 (20 μg/mL). At indicated time points (1, 4, 24 hours), 30 μL aliquots were collected and analyzed with gelatin zymography. TSP-1 induced more pro-MMP2 activation at 1 and 4 hours compared with their respective negative controls.

a basement membrane substrate. In the first experiment, VSMC monolayer cultures on uncoated plates were exposed to TSP-1 for 1, 4, or 24 hours. The resultant conditioned media were analyzed with gelatin zymography. In the second experiment, VSMC monolayer cultures on uncoated plates were exposed to TSP-1 for 1, 4, or 24 hours. To specifically show upregulation by TSP-1 of steady-state mRNA for MMP2, the cells were used for Northern blot analysis. To determine whether TSP-1 concentration was important for MMP2 mRNA upregulation, 10 and 20 μg/mL concentrations were used, as well as SFM (negative control) and 10 ng/mL platelet-derived growth factor (PDGF-BB, positive control, 1-hour exposure time).

Invasion assays. In assay 1, membranes were coated with Matrigel. TSP-1, SFM (negative control), or tumor necrosis factor-α (TNFα; positive control) was in the top chamber, with VSMCs and 10% FBS in the bottom chamber. The assay was run for 18 hours. In assay 2, the medium from the top chamber in invasion assay 1 was used for activation experiments with gelatin zymography. In assay 3, to distinguish the importance of MMPs in invasion (penetration of basement membrane) versus chemotaxis, invasion assays (Matrigel-coated membrane) and chemotaxis experiments (uncoated membrane) were performed with the MMP inhibitor GM6001. This assay was run for only 4 hours, a standard time for running chemotaxis assays.

Binding assays. The purpose of these experiments was to demonstrate whether activated MMP2 is bound to TSP-1 and whether the degree of binding is similar or different from that of other ECM proteins. First, plates were coated with BSA, TSP-1, or type I collagen. Activated MMP2 was added, incubated for 24 hours, and soluble and bound protein was retrieved and used for both gelatin zymography and Western blot analysis. Second, after demonstrating that activated MMP2 binds TSP-1, TSP-1 was compared with elastin, collagen I, and fibronectin. Bound protein was retrieved, and comparisons were made with gelatin zymography.

RESULTS

TSP-1 upregulates pro-MMP2. To investigate the role of MMP2 in TSP-1–mediated VSMC migration, the effect of TSP-1 on MMP2 production was determined. In the resultant conditioned media, analyzed with gelatin zymography, the SFM group showed increased production of 72 kDa pro-MMP2, which was time-dependent (Fig 1). TSP-1 induced more pro-MMP2 activation at 1 and 4 hours in comparison with SFM. MMP9 was not expressed.

TSP-1 increases MMP2 activation during VSMC invasion. Invasion assays were performed to identify whether the migratory activity of TSP-1–stimulated VSMCs through a matrix protein substrate is regulated by gelatinase activity. After 18 hours, media in the top chamber showed MMP2 activation in the TSP-1 and TSP-1/TNF-α groups (Fig 2, *A*). A small amount of 72 kDa pro-MMP2 was noted in these two groups as well. Lighter bands noted between 59 and 72 kDa likely represent intermediate forms of MMP2. This finding is consistent with other studies.[18,19] TSP-1, with or without TNF-α, showed more migration than did SFM through the Matrigel-coated membrane toward 10% FBS (TSP-1, 156 ± 5.8 cells/5 HPF [±SEM]; TSP-1/TNF-α, 167 ± 7.5; SFM, 46 ± 9.1; $P < .05$; Fig 2, *B*). TSP-1, with or without TNF-α, showed a similar amount of cell migration. These results suggest that TSP-1 could modulate MMP2 activity during VSMC migration.

TSP-1 increases MMP2 mRNA levels. More pro-MMP2 was expressed in the conditioned media of VSMCs with TSP-1 when compared with SFM (Fig 1). To examine this mechanism, MMP2 mRNA was measured in VSMCs after exposure to TSP-1 for 1 hour. TSP-1 (10 or 20 μg/mL) and the positive control, platelet-derived growth factor BB (PDGF-BB), induced MMP2 mRNA expression (1.7 ± 0.29; $P < .05$; Fig 3, *A, B*). MMP2 gene activation to TSP-1 was time-dependent (Fig 3, *C*).

MMPs are required for TSP-1–induced VSMC chemoinvasiveness. The hypothesis that MMPs are important in TSP-1–induced VSMC invasion through a matrix barrier was tested with Matrigel-coated membranes. With the MMP inhibitor (GM6001), the effective role of MMPs in the invasive activity of these cells was demonstrated. Invasion was abolished by using GM6001 with TSP-1–treated cells (66.67 ± 2.6 vs 17.67 ± 2.96 cells/5 HPF [±SEM]; $P = .0075$; Figs 4 and 5, *A*). GM6001 did not affect the cells morphologically (Fig 4). Placing GM6001 in the bottom well did not inhibit TSP-1–induced chemoinvasiveness. No change was noted in chemotaxis of VSMCs with or without the MMP inhibitor when an uncoated membrane was used (Fig 5, *B*). These results

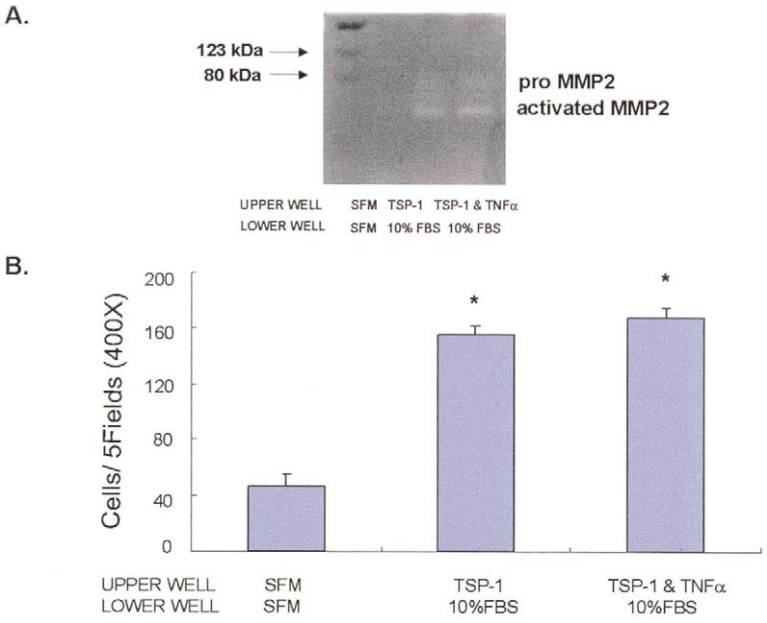

Fig 2. TSP-1 increases MMP2 activation during VSMC invasion. **A,** A modified Boyden chamber was used. The porous membrane separating the two chambers was coated with Matrigel. VSMCs were stimulated with TSP-1 (20 μg/mL) or TSP-1 with TNF-α (10 ng/mL) in the upper well (18 hours) and 10% FBS in the lower well (chemoattractant). Zymography demonstrated gelatinolytic activity of MMP2 in media obtained from the upper well in the TSP-1 with or without TNF-α groups. **B,** Findings are summarized for a representative invasion assay (N = 3). TSP-1 with or without TNF-α induced more invasion than SFM alone ($P < .05$). No significant difference was noted between TSP-1 and TSP-1 with TNF-α. *$P < .05$ in comparison with negative control (SFM).

suggest a specific role for MMP2 in cell-matrix barrier invasion by TSP-1–stimulated VSMCs.

TSP-1 binds MMP2. To evaluate the role of TSP-1 binding in regulation of MMP2 activity, interaction of MMP2 and TSP-1 was studied. Microtiter plates coated with BSA (negative control), TSP-1, or type I collagen (positive control) were incubated with MMP2 overnight, and qualitative analysis of matrix-bound MMP2 was demonstrated with gelatin zymography (Fig 6, *A*) and Western blot (Fig 6, *B*). Active MMP2 was bound to TSP-1 and type I collagen–coated wells, but not in BSA-coated control samples.

TSP-1 preferentially binds MMP2 over other ECM proteins. The relative catalytic activity of bound MMP2 was assessed for TSP-1, elastin, type I collagen, and fibronectin. With zymography there was more catalytic activity by TSP-1 than the other proteins studied (Fig 7). This finding suggests that TSP-1 is a specific matrix protein to induce MMP2 activation.

DISCUSSION

Migration of VSMCs and remodeling of ECM are significant events in development of intimal hyperplasia and atherosclerosis.[6] TSP-1 is an ECM protein, relevant to different important processes, eg, vessel remodeling after acute arterial injury, and in development of atherosclerotic plaque and intimal hyperplasia.[20] MMP2 is an important protein for VSMC migration by ECM remodeling and is activated in balloon catheter arterial injury lesions.[21,22] Therefore we hypothesized that TSP-1 can modulate MMP2 activity during VSMC invasion or cell migration with penetration of the basement membrane by VSMCs. No established information is available for MMP2 and TSP-1 for VSMCs, although several conflicting reports exist regarding the interaction between MMP2 and TSP-1 in endothelial cells undergoing angiogenesis.[23,24]

First, MMPs are relevant for TSP-1–induced VSMC invasion. TSP-1–stimulated VSMCs could invade a Matrigel-coated filter as they migrated toward the chemoattractant 10% FBS. A significant number of cells adhered to the Matrigel-coated filters and migrated to the underside of the membrane in the direction of the chemoattractant. The invasive ability of these VSMCs was reduced significantly when MMP activity was inhibited with GM6001. This inhibition was specific for cell invasion, inasmuch as chemotaxis induced by TSP-1 was unaffected by GM6001.

The major gelatinase expressed in vitro by VSMCs in response to the multifunctional matricellular protein TSP-1 was MMP2. MMP2 was present in TSP-1–conditioned media in both the latent (pro-MMP2) and activated (MMP2) forms. MMP2 expression is highly regulated by environmental factors, and it also digests a number of

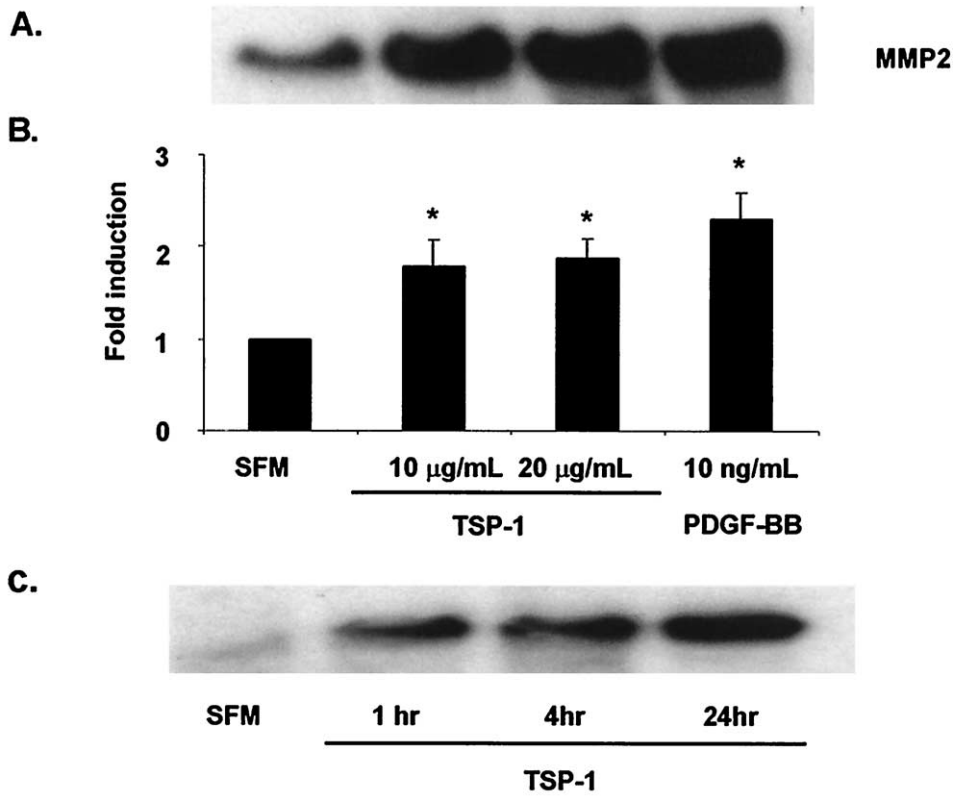

Fig 3. TSP-1 increases MMP2 mRNA levels. **A,** Representative Northern blot demonstrates that after 1 hour of exposure TSP-1 (10 and 20 μg/mL) and PDGF-BB (10 ng/mL) induce upregulation of MMP2 steady-state mRNA. **B,** Densitometric analysis of three independent experiments demonstrates that upregulation of MMP2 steady-state mRNA occurred in groups exposed to TSP-1 (10 or 20 μg/mL) or PDGF-BB (10 ng/mL). There was no significant difference for degree of upregulation by the three groups in comparison with SFM alone. *$P < .05$ in comparison with negative control (SFM). *C,* Representative Northern blot indicates that TSP-1–induced upregulation of MMP2 mRNA increases over the times measured (1, 4, 24 hours).

matrix proteins, including fibronectin, laminin, and collagen type V.[25] MMP2 is different from other MMPs, and it is unaffected by a number of physiologic stimulators. For example, a number of endopeptidases that stimulate MMP1 and MMP3 do not activate MMP2.[25]

One reason why MMP2 may be activated by TSP-1 relates to the interaction between TSP-1 and transforming growth factor-β_1 (TGF-β_1). Previous studies reported that TGF-β_1 regulates MMP2 expression at the transcription and posttranscription levels by inducing early increase in MMP2 transcription and increase in the half-life of MMP2 mRNA.[26] Data by other investigators indicate that TSP-1 can bind and is an important regulator for activation of TGF-β_1.[27-29] We speculate that MMP2 activation by TSP-1 may be due to the indirect effect of TGF-β_1 activation.

Like many MMPs, MMP2 is secreted as a latent proenzyme, pro-MMP2, and must be activated extracellularly. The cellular mechanism for pro-MMP2 activation is complicated and must be controlled carefully, and activation occurs only during certain processes, eg, cell migration or invasion. Activation of secreted pro-MMP2 occurs at the cell surface by way of formation of a ternary complex between pro-MMP2, tissue inhibitor of metalloproteinase-2 (TIMP-2), and a transmembrane MMP designated membrane type I MMP (MT1-MMP).[30,31] Active MMP2 undergoes concentration-dependent autolysis, which results in cleavage of the hemopexin-like domain from the catalytic domain.[32] This observation suggests that the active state of MMP2 is unstable in the absence of substrate and indicates that autolytic inactivation may function as an alternative mechanism for the posttranslational control mechanism of MMP2 activity. For example, type I and type IV collagen promoted MMP2 activation in a dose-dependent manner through stabilization of MMP2 against an autolytic mechanism.[16,33] The role of TIMP-2 and MT1-MMP in TSP-1–induced MMP2 activation in VSMCs has not been defined and will be studied in our laboratory.

TSP-1 activates MMP2 in VSMCs. MMP2–TSP-1 interactions were evaluated to determine whether MMP2 binding to TSP-1 serves as a positive regulator of MMP2

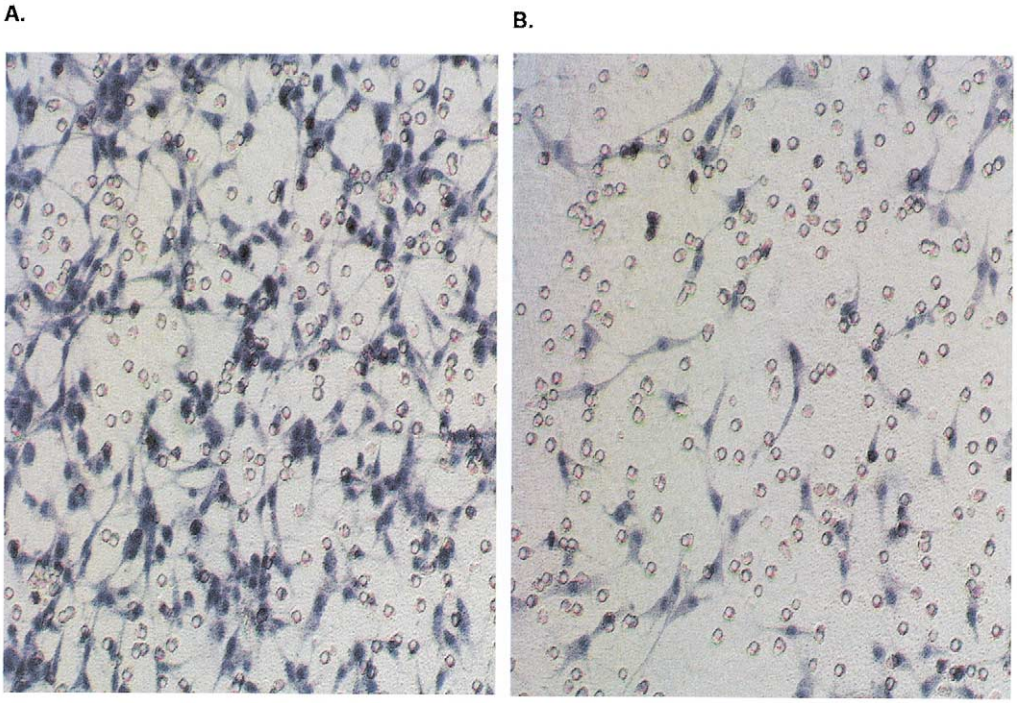

Fig 4. GM6001 inhibits TSP-1–induced VSMC invasion of membrane. **A,** TSP-1–stimulated VSMCs (5×; 10^5), suspended in DMEM containing 0.1% BSA, were added to upper chamber. Migrated VSMCs through Matrigel were stained with hematoxylin (200×). **B,** GM6001 (MMP inhibitor)–pretreated VSMCs were used instead in upper chamber. Fewer cells migrated in the GM 6001–pretreated group.

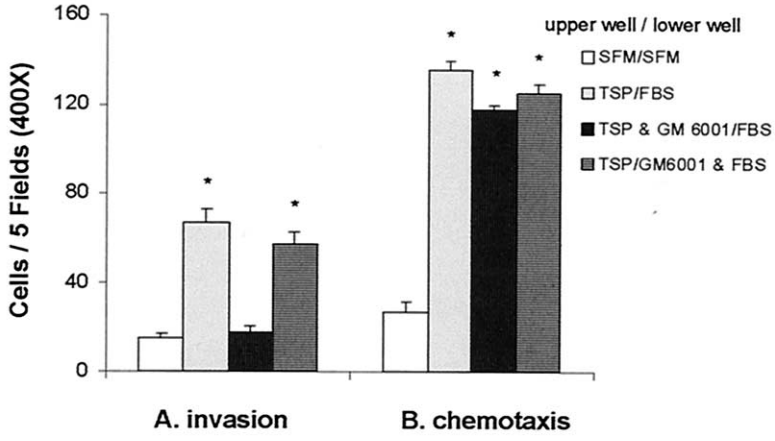

Fig 5. MMPs are required for TSP-1–induced chemoinvasiveness, not chemotaxis. Bar graphs show effect of GM6001 on chemoinvasion and chemotaxis of TSP-1–stimulated VSMCs. **A,** VSMCs with or without GM6001 were placed in top chamber containing TSP-1. Bottom chamber contained 10% FBS with 0.1% BSA. Membrane separating the two chambers was coated with Matrigel. Presence of GM6001 in the upper well prevented invasion of TSP-1–stimulated VSMCs. GM6001 in the bottom chamber had no significant effect on invasion. $*P < .05$ in comparison with respective negative controls. **B,** Chemotaxis was measured in absence of a matrix barrier. MMP inhibition (GM6001) did not affect VSMC chemotaxis.

activation. The results showed that MMP2 was detected in TSP-1–coated plates. This finding is consistent with other reports that TSP-1 interacts directly with MMP2.[23,34] Furthermore, in comparison with other ECM proteins studied, ie, type I collagen, fibronectin (structural glycoprotein), and elastin, TSP-1–bound MMP2 showed elevated activity

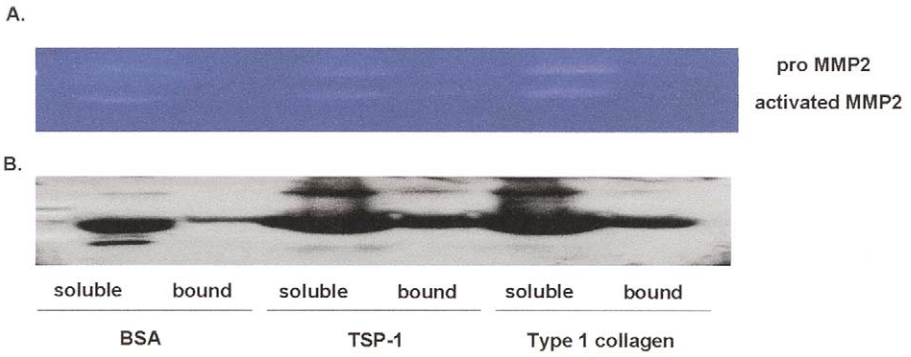

Fig 6. TSP-1 binds MMP2. MMP2 (50 nmol/L) was pretreated with amino-phenylmercuric acetate and incubated overnight (4°C) in wells coated with BSA, TSP-1, or type I collagen (positive control, 50 μg). Soluble enzyme was removed (designated "soluble"). After washing wells with assay buffer, bound enzyme was eluted with two different techniques: bound enzyme was eluted with nonreducing Laemmli sample buffer (50 μg, designated "bound") and analyzed with gelatin zymography; and bound enzyme was eluted with reducing Laemmli sample buffer (50 μg, designated "bound") and analyzed with Western blot. Both techniques demonstrated that TSP-1 binds MMP2. BSA, TSP-1, and type I collagen upregulated pro-MMP2 and MMP2 when analyzing the soluble forms.

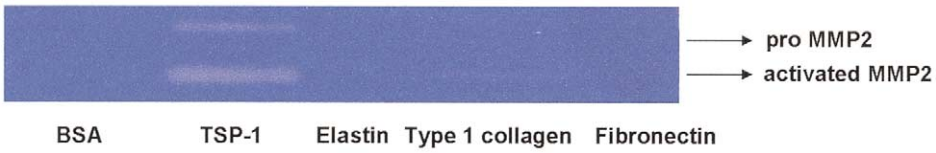

Fig 7. TSP-1 preferentially binds MMP2. To quantify activity of MMP2 bound to ECM proteins, MMP2 (10 nmol/L) was incubated overnight (4°C) in wells coated with BSA, TSP-1, elastin, type 1 collagen, or fibronectin. After analysis, MMP2 was eluted with reducing Laemmli sample buffer (50 μg, designated "bound") and analyzed with gelatin zymography. TSP-1 had a stronger binding affinity for MMP2 than did other ECM proteins studied.

at zymography. We speculate that TSP-1 has a stronger affinity than the other matrix proteins for MMP2, which might affect autolytic inactivation of MMP2.

In summary, TSP-1 induces MMP2 activity through transcriptional or posttranslational mechanisms that are related intimately to VSMC invasion. Results of this study suggest that MMP2 activation is relevant to VSMC invasion, an important in vivo process in development of vascular lesions. This study establishes a preliminary relationship between TSP-1 and MMP2. Future studies should define which receptor site or sites of TSP-1 for VSMCs are responsible for activation of MMP2; determine the role of MT-1-MMP and TIMP2; and determine whether the effect of TSP-1 on MMP2 activation is the result of increased activation or, to some extent, decreased autolysis of activated MMP2.

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